Short-Term Intermittent Hypoxia Improves Stroke Outcome in Mice

Honors Research Thesis

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Abstract

Obstructive sleep apnea (OSA) is a disorder characterized by airflow interruptions during sleep, and has been linked to increased incidence of stroke. Intermittent hypoxia (IH) in experimental animals can partially recapitulate the physiology of OSA. Previous studies have indicated that IH stimulates protective mechanisms in the brain that can protect against stroke. This protection is termed, preconditioning; the temporal requirements of IH treatment that produce preconditioning are not known. To explore the potential role of short-term versus chronic IH in stroke outcome, we analyzed infarct volume and expression of proinflammatory cytokine gene expression (*TNF-α, IL-6* and *IL-1β*) after stroke in mice treated with IH or room air for 11 or 20 days.

Mice experiencing IH for 11 days prior to stroke and 1 day after reperfusion decreased gene expression of both *IL-6* and *IL-1β*. When assessed 3 days post-stroke infarct volume was decreased in mice that received IH prior to stroke and room air after stroke compared to mice treated with air prior to and after stroke. These results were not observed in mice that experienced IH 20 days prior to stroke and 3 days after stroke. These results suggest that short-term IH prior to stroke is protective and reduces both infarct volume and inflammatory gene expression compared to long-term IH prior to stroke.

Introduction

Obstructive sleep apnea (OSA) is a condition in which airflow is repetitively decreased or stopped while sleeping. OSA is highly prevalent in the population and leads to significant morbidity, even in mild cases[1]. Specifically, OSA is known to be associated with increased likelihood of stroke[1]. Stroke is the third-leading cause of death and the leading cause of long-term disability in the Western world[2]. During ischemic stroke the brain is deprived of oxygenated blood and nutrients leading to necrosis of neural tissue. The resulting damage to neuronal tissue can be devastating and produce lasting sensorimotor deficits. Although it is unclear whether OSA is an independent risk factor for stroke or whether it takes effect only in the presence of other traditional cardiovascular risk factors, there is certainly an association between the two that should be investigated[3]. The current predominant treatment for stroke is thrombolysis, but many individuals with a high risk for stroke might instead benefit from therapies that improve the brain's resistance to ischemic injury prior to stroke onset[4].

Ischemic stroke results in necrosis of tissue, or infarcted tissue, in the immediate area in which the insult occurred. This damage incurred after stroke is two-fold. Initially there is damage from apoptotic cell death resulting from oxygen deprivation during the acute focal ischemia[5]. That damage reflects how long and to what extent the vessel in the brain was occluded. Secondary damage is caused by inflammatory processes incited by microglia present in the central nervous system that takes several days to develop after the initial injury[6]. This presents a

therapeutic window during which interventions may improve the outcome for patients. One way to quantify damage after stroke is infarct volume, which measures the amount of necrotic tissue post-stroke. Another way to evaluate the brain post-stroke is by measuring the presence of certain inflammatory cytokines. Microglia release and respond to many inflammatory cytokines such as interleukin-6 (*IL-6*), interleukin 1-beta (*IL-1β*), and proinflammatory tumor necrosis factor alpha (*TNF-α*)[7]. Following stroke the expression of these cytokines are up-regulated, facilitating inflammatory cascades which cause further cerebral infarction[5]. Although some neuroinflammation is key to recovery, failure to regulate these processes can lead to additional damage in the days following stroke[8].

Intermittent hypoxia (IH), or repetitive cycling between hypoxia and reoxygenation, can be utilized as a model for OSA in mice because animals undergoing IH treatment exhibit blood oxygen levels consistent with OSA patients[9]. Animal models of IH have also shown changes in blood pressure consistent with those observed in OSA patients[10]. OSA has been associated with increased mortality following stroke[11]. Therefore, it is plausible to suggest that IH may universally cause an increase in inflammatory cytokines *IL-6*, *IL-1β*, and *TNF-α* leading to more severe damage after stroke. However, this may depend on the duration of IH exposure because IH is observed to have differing effects depending on exposure length. Exposure to IH lasting up to 14 days is generally considered acute while anything longer is considered chronic. Exposure to acute IH appears to stimulate neurogenesis and reduces depressive-like behavior[12,13], but is also associated with increased *TNF-α* gene expression[14]. However, chronic IH is associated with impaired

cognitive function, increased inflammation and oxidative stress[9]. In a study utilizing IH as a model for OSA, increased levels of pro-inflammatory cytokines were present in animals receiving IH treatment[15]. Expression of *IL-6* and *IL-1β* are known to increase after arterial occlusion and facilitates the inflammatory process by mediating inflammatory cascades[5]. Therefore a mechanism that decreases expression of these inflammatory cytokines would also decrease damage after stroke. Preclinical models have demonstrated that exposure to hypoxia is generally protective for only a few days, however a repetitive hypoxic environment can extend the preconditioning effect of hypoxia in stroke for up to 2 months after completion of IH treatment[4]. However, it is still unknown how longer durations of IH will affect stroke outcome.

Problem Identification and Justification

Previous literature has demonstrated patients with OSA are at an increased risk for stroke[16]. It is therefore important and relevant to investigate the disparity between the apparent preconditioning role for IH in stroke and the known relationship between OSA and increased incidence of stroke. A potential explanation could be the length of exposure to IH, because most patients with OSA do not experience the effects of IH over a short period of time. As mentioned, IH has differing effects depending on duration of exposure. Since studies that observed a preconditioning effect for IH in stroke involved administering treatment for a brief duration, this research investigated the effects of IH for longer time each day and

over many days. The study aimed to determine whether short-term IH (11+3) or chronic IH (20+3 days) experienced for 8 h/day also has a preconditioning role.

Hypothesis

I hypothesize that infarct volume will increase proportionally across the treatment groups (Table 1). Infarct volume should be smallest in the air/air treatment groups and largest in the IH/IH treatment groups. I also hypothesize that this will be mirrored by decreased expression of inflammatory markers (*IL-6*, *IL-1β*, *TNF-α*) in the stroke hemisphere. I expect the results to indicate that IH does not have a preconditioning role against stroke when exposure occurs for a longer duration (20 days).

Materials and Methods

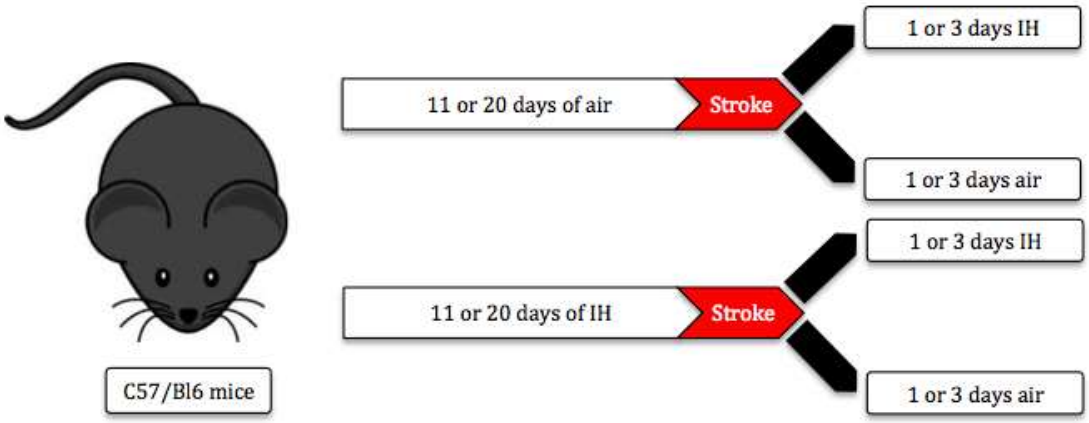


Figure 1. Diagram of short vs. long-term groups. Mice received either 11 (short-term) or 20 (long-term) days of air or IH treatment, were subjected to a stroke, and were then placed back into treatment (IH or air) for 1 or 3 days.

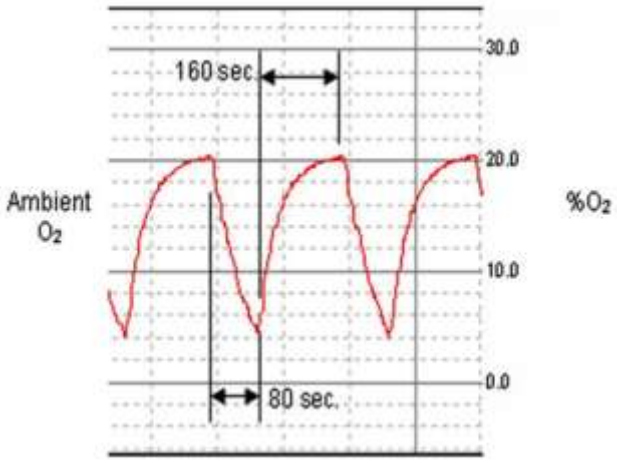


Figure 2. Oxygen levels fluctuated in the IH chamber between 21% and 5%.

	Pre-stroke Treatment	**Post-stroke Treatment**
Group 1	**Air**	**Air**
Group 2	**IH**	**Air**
Group 3*	**Air**	**IH**
Group 4	**IH**	**IH**

Table 1. Treatment groups; two sets of animals for infarct volume short-term (11+1 days and 11+3 days), one set for infarct volume long-term (20+3 days), and a third set for gene expression short-term (11+1 days). *Group 3 was omitted in the short-term study (11+3 days).

Animals

Adult male C57/Bl6 (> 8 weeks, ~ 23g) mice were acclimated to the facility for one week. Mice were group housed in a 14:10 light/dark cycle on ventilated racks in a temperature- and humidity- controlled vivarium with *ad libitum* access to food (Harlan 8640 Teklad Rodent Diet) and filtered tap water.

Intermittent Hypoxia

Mice were then exposed to either 11 or 20 days of room air or IH (15 cycles/hr, 8hr/day, FIO_2 nadir of 5%) (Figure 1). Eleven days of pre-stroke treatment refers to the short-term model, and 20 days of pre-stroke treatment refers to the long-term model. During this treatment mice were moved to custom-designed Plexiglas chambers (31 cm x 19 cm x 18 cm) with a raised floor (6.5 cm) during the light cycle and moved into home cages in a separate room during the dark cycle. Twelve mice were placed in one chamber at a time. Oxygen levels were controlled by connecting the cages via a regulator system to compressed air and nitrogen tanks. The oxygen levels (21%- 5%) cycled throughout the day (Figure 2). Fluctuation in chamber oxygen levels was achieved by alternating 3 min of breathing air and 1 min of nitrogen. Mice exposed to room air were housed in a similar cage, without connections to nitrogen or air tanks. Treatment occurred during the light phase (when these animals typically sleep) to mimic when OSA would occur in patients.

Middle Cerebral Artery Occlusion

On the twelfth or twenty-first day mice were subjected to a middle cerebral artery occlusion (MCAO), which causes transient focal cerebral ischemia[17]. Briefly, mice were anesthetized with isoflurane in oxygen, and unilateral right MCAO was achieved by insertion of a 6-0 nylon monofilament into the internal carotid artery to a point 6 mm beyond the internal carotid-pterygopalatine artery bifurcation. After the monofilament was secured, the wound was sutured. Occlusion occurred for 60 min, during which mice were allowed to recover from anesthesia. After 60 min the animal was re-anesthetized and reperfusion was initiated by removal of the filament. Animals were then allowed to recover until treatment the next morning. The following day animals were placed back in either air or IH treatment for 1 or 3 days. See Figure 1 for a flowchart depicting the different treatments.

Tissue Collection and Processing

After the conclusion of treatment animals were anesthetized with isoflurane vapors and euthanized via rapid cervical dislocation and decapitation. Brains were rapidly removed, frozen on dry ice for 5 min, and then sectioned into five 2-mm-thick coronal sections. Sections were processed with 2,3,5- triphenyltetrazolium chloride (TTC) and incubated for 12 min in a water bath held at 37° C. TTC is processed by living mitochondria and turns the living tissue red while dead tissue remains white, allowing comparison of infarct volume across groups. Following staining sections were fixed in formalin for at least 24 h. Then brain slices were photographed and analyzed using Inquiry software (Loats Associates, Inc.,

Westminster, MD). Infarct size was calculated as a percentage of the contralateral hemisphere after correcting for edema using the following formula: [(1-(total ipsilateral hemisphere-infarct))/total contralateral hemisphere] *100.

One cohort of mice receiving the 11 day pre- stroke/1 day post-stroke treatment was used for gene expression. Mice were euthanized during the light phase, and brains were collected and placed in RNAlater (Applied Biosystems, Foster City, CA). After > 24 hr the cortex was dissected out for PCR. The RNA was extracted using a handheld homogenizer (Fisher Scientific, Waltham, MA) and TRIzol Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's guidelines. cDNA was reverse transcribed from 2 □g RNA with MMLV Reverse Transcriptase enzyme (Invitrogen, Carlsbad, CA) according to the manufacturer's guidelines. Proinflammatory cytokine expression of *IL-1β, IL-6, and TNF-*□ were determined using primer and probe assays kits (Applied Biosystems, Foster City, CA) on an ABI 7500 Fast Real Time PCR System using Taqman Universal PCR Master Mix. The universal two-step RT-PCR cycling conditions used were: 50° C for 2 min, 95° C for 10 min, followed by 40 cycles of 95° C for 15 s and 60° C for 1 min. Relative gene expression of samples run in duplicate were calculated based on a relative standard curve and standardized to 18s rRNA signal.

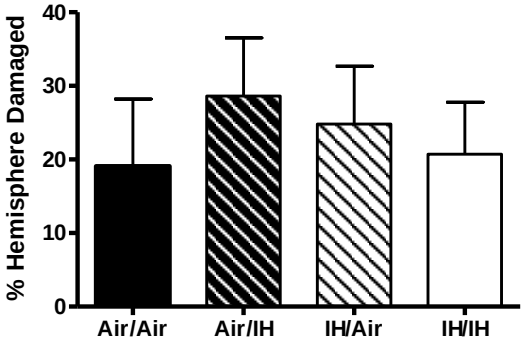
Statistical Analysis

Infarct volumes were compared using T-tests to assess planned comparisons between groups. Cytokine expression was analyzed with Kruskal-Wallis test due to unequal variances among groups, and followed up by Mann-Whitney U post hoc tests assessing effects of pre and post MCAO treatment (room air or IH) and hemisphere (ipsilateral and contralateral). Differences were considered significant at $p<0.05$, and were followed up with post-hoc tests. Statistics were conducted using SPSS 19 for Windows (IBM, Armonk, New York, USA).

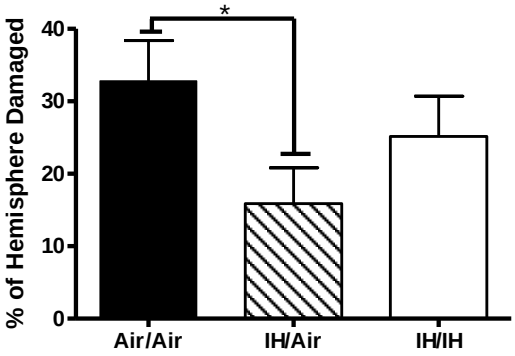
Results

Infarct Volume

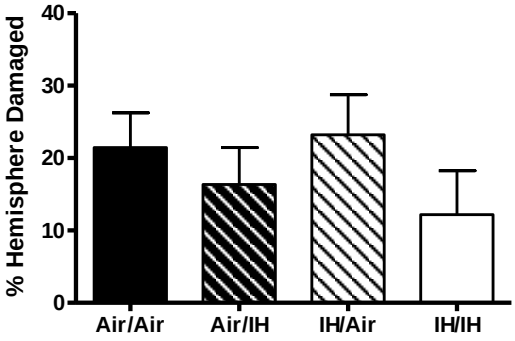
When analyzed 1 day after MCAO in the 11-day pre-stroke model, no differences were observed in percent infarct between treatment groups ($p>0.05$). When analyzed 3 days after MCAO in the 11-day pre-stroke model, mice that received IH prior to and air following MCAO (M=15.89, SD=13.64) had reduced percent infarct compared to mice exposed to only room air (M=32.80, SD=17.04); $t(14)=2.21$, $p=0.044$). In the same model, mice exposed to only IH (M=25.15, SD=13.71) displayed similar percent infarct compared to mice exposed to IH only prior to MCAO (M=15.89, SD=13.64) and mice exposed only to room air (M=32.80, SD=17.04, $p>0.05$). When analyzed 3 days after MCAO in the 20-day pre-stroke model, no differences were observed in percent infarct among groups ($p>0.05$). (See Figure 3A-3C)



3A. 12 Day Exposure (11 Days Pre Stroke, 1 Day Post Stroke)



3B. 14 Day Exposure (11 Days Pre Stroke, 3 Days Post Stroke)

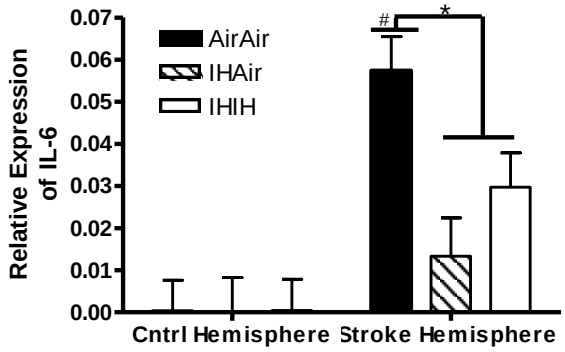


3C. 23 Day Exposure (20 Days Pre Stroke, 3 Days Post Stroke)

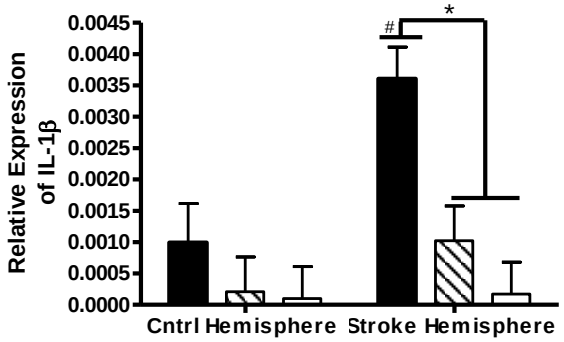
Figure 3. Infarct volume was reduced in mice experiencing IH prior to stroke. Percent of hemisphere damaged one day following middle cerebral artery occlusion (MCAO) was similar among groups exposed to 11 days of treatment prior to MCAO (A). Percent of hemisphere damaged three days following MCAO was reduced in mice exposed to 11 days of IH prior to MCAO (B). Percent of hemisphere damaged three days following MCAO was similar among groups exposed to 20 days of treatment prior to MCAO (C). *indicates significant differences at $p<0.05$.

Inflammatory Gene Expression

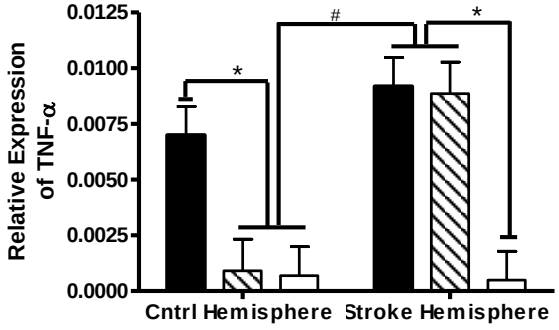
Tissue was collected for inflammatory gene expression in the short-term 11+1 day model. Following one day of treatment after MCAO *IL-6* gene expression was increased in the ipsilateral hemisphere compared the contralateral hemisphere (U(1)=12, Z= -4.25, $p<0.05$). *IL-6* was increased in the ipsilateral hemisphere of air/air treated mice compared to IH/air treated mice (U(1)=1, Z= -2.21, $p<0.05$). *IL-1β* gene expression was increased in the ipsilateral hemisphere compared to the contralateral hemisphere (U(1)=58, Z= -2.63, $p<0.05$). *IL-1β* was increased in the ipsilateral hemisphere of air/air treated mice compared to IH/IH treated mice (U(1)=0, -2.88, $p<0.05$). *IL-1β* was increased in the ipsilateral hemisphere of air/air treated mice compared to IH/IH treated mice (U(1)=0, -2.88, $p<0.05$). *TNF-α* gene expression was increased in both hemispheres in mice exposed to air throughout the entire experiment compared to mice exposed only to IH (U(1)=0, Z=-2.88, $p<0.05$). Mice exposed to IH prior to stroke and air after stroke increased *TNF-α* gene expression only in the ipsilateral hemisphere (U(1)=1, Z=-2.40, $p<0.05$). *TNF-α* gene expression was similar in both hemispheres in mice exposed only to IH ($p>0.05$). (See Figure 4A-4C)



4A. Gene expression of *IL-6*



4B. Gene expression of *IL-1β*



4C. Gene expression of *TNF-α*

Figure 4. Inflammatory gene expression was reduced in mice experiencing IH prior to stroke. Relative gene expression of *IL-6* (A) and *IL-1β* (B) was decreased one day following MCAO in mice exposed to IH compared to mice exposed only to air. Relative gene expression of *TNF-α* (C) was decreased one day following MCAO in mice exposed to IH prior to stroke and air after stroke only in the ipsilateral hemisphere. * indicates significant differences within a hemisphere, # indicates significant differences from all other groups in contralateral hemisphere, & indicates significant differences in the same group between the ipsilateral and contralateral hemispheres at $p < 0.05$.

Discussion

OSA is associated with increased risk for stroke, and can be modeled by intermittent hypoxia (IH) treatment in mice[1,9]. Previous literature indicates a preconditioning role for short-term IH in stroke, but the effect of long-term IH is still undetermined[4]. Therefore we subjected mice to both short- (11 days) and long- (20 days) term IH treatment and observed the effect each of these treatments had on stroke outcome assessed by infarct volume and inflammatory gene expression of *IL-6, IL-1β* and *TNF-α*. The results indicated a preconditioning effect of short-term IH when analyzed 1 day after stroke because infarct volume and inflammatory gene expression were both significantly decreased compared to other groups. This preconditioning effect was absent in the long-term IH model.

The differences observed between analyzing infarct 1 or 3 days after stroke suggest that a mechanism of penumbral sparing is at work. In ischemic stroke, there are two areas of injury – the core, and the penumbra. The core represents the damage from the initial insult, and constitutes dead tissue that cannot be saved. The penumbra, while at risk for becoming infarct, still has the potential to be saved. The penumbra represents the area surrounding the core injury and is the area of focus when trying to improve stroke outcome[18]. Infarct volume in mice takes 3 days to fully develop as the penumbra is saved or the core expands[19]. Therefore, observing differences 3 days after stroke but not 1 day after stroke indicates that after 1 day only the core injury is being measured, and that the penumbral tissue is still alive though at risk. After 3 days, the observed decline in infarct volume in animals

treated with IH indicates IH played a role in sparing the penumbra in those animals. Additionally, the data indicate that this protective effect is not present when exposed to a long-term duration of IH.

The results for *IL-6* and *IL-1β* also suggest a mechanism of penumbral sparing because inflammation, while beneficial in many cases, can cause further damage after stroke if it is too excessive[8]. Therefore, observing decreased expression of *IL-6* and *IL-1β* only in animals that received IH treatment indicates IH plays a role in limiting inflammation after a stroke. The observed increase in *TNF*-α may also be linked to protective effects because it is only seen in the hemisphere that received the stroke. This indicates the inflammatory response is more localized and is lessened. Whereas mice exposed to air throughout the experiment had increases in *TNF-α* gene expression in both hemispheres, suggesting dysregulated control of the inflammatory response. Gene expression was analyzed 1 day after stroke in the 11-day exposure model. Observing decreases in *IL-6* and *IL-1β* expression at 1 day after stroke but not observing changes in infarct volume at this point indicates that the decreased inflammatory response at 1 day after stroke sets up an environment in which there will be controlled inflammation to allow more tissue sparing at three days following stroke.

Conclusion

Short-term exposure to intermittent hypoxia has a preconditioning effect on stroke in mice. Decreased expression of inflammatory genes *IL-6* and *IL-1β* along with the observation of decreased infarct volume when assessed 3, but not 1 day post stroke together suggest a mechanism of penumbral sparing by decreased inflammation in the brain. Intermittent hypoxia before stroke sets up an environment in which there will be controlled inflammation to allow sparing of the penumbra. However, long-term exposure to IH does not protect against stroke damage. These results have potential implications for deriving clinical therapies of short-term IH exposure in individuals at high risk for stroke.

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